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Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms

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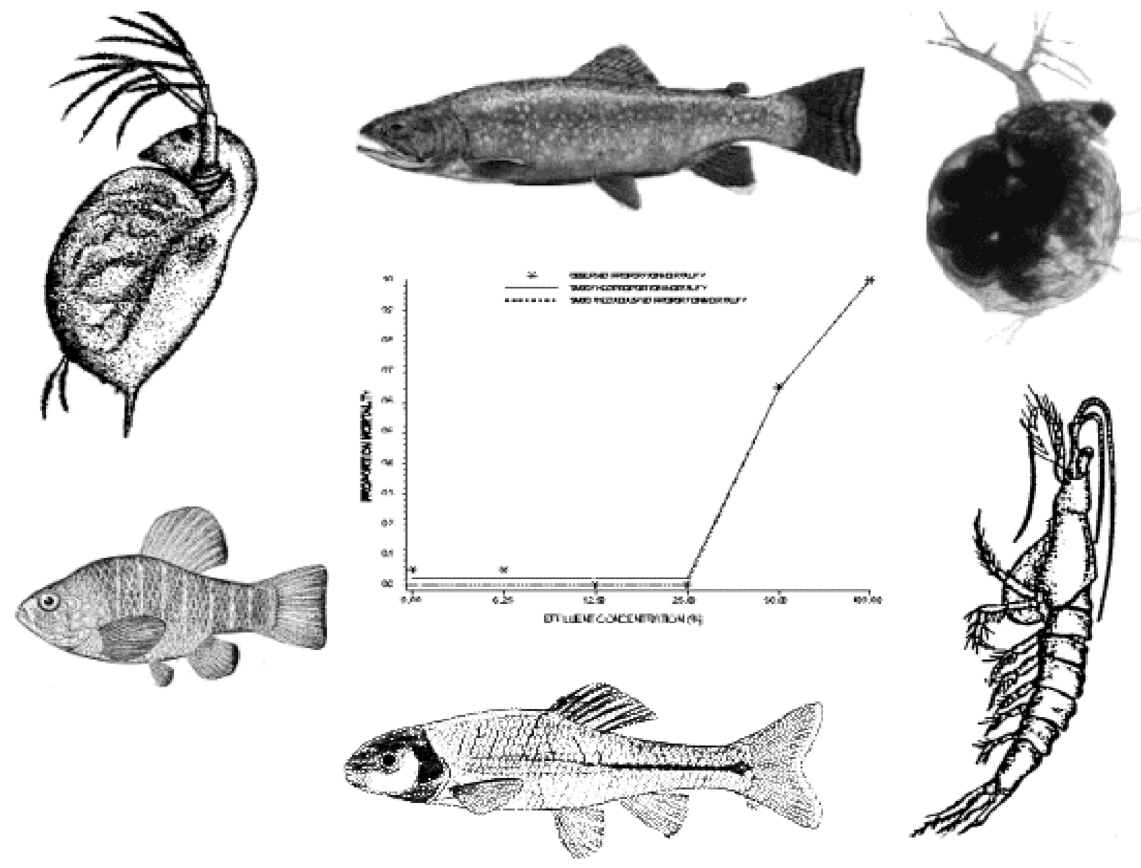

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SECTION 1

INTRODUCTION

1.1 This manual describes acute toxicity tests for use in the National Pollutant Discharge Elimination System (NPDES) Permits Program to identify effluents and receiving waters containing toxic materials in acutely toxic concentrations. With the exception of the *Holmesimysis costata* Acute Test (Table 19), the methods included in this manual are referenced in Table IA, 40 CFR Part 136 regulations and, therefore, constitute approved methods for acute toxicity tests. They are also suitable for determining the toxicity of specific compounds contained in discharges. The tests may be conducted in a central laboratory or on-site, by the regulatory agency or the permittee. The *Holmesimysis costata* Acute Test (Table 19) is specific to Pacific Coast waters and is not listed at 40 CFR Part 136 for nationwide use. This method has been proposed but not yet approved at 40 CFR Part 136.

1.2 The data are used for NPDES permits development and to determine compliance with permit toxicity limits. Data can also be used to predict potential acute and chronic toxicity in the receiving water, based on the LC50 and appropriate dilution, application, and persistence factors. The tests are performed as a part of self-monitoring permit requirements, compliance biomonitoring inspections, toxics sampling inspections, and special investigations. Data from acute toxicity tests performed as part of permit requirements are evaluated during compliance evaluation inspections and performance audit inspections.

1.3 Modifications of these tests are also used in toxicity reduction evaluations and toxicity identification evaluations to identify the toxic components of an effluent, to aid in the development and implementation of toxicity reduction plans, and to compare and control the effectiveness of various treatment technologies for a given type of industry, irrespective of the receiving water (USEPA, 1988a; USEPA, 1988b; USEPA, 1989a; USEPA, 1989b; USEPA, 1991a).

1.4 This methods manual serves as a companion to the short-term chronic toxicity test methods manuals for freshwater and marine organisms (USEPA, 2002a; USEPA, 2002b), the NPDES compliance inspection manual (USEPA, 1988c), and the manual for evaluation of laboratories performing aquatic toxicity tests (USEPA, 1991b). In 2002, EPA revised previous editions of each of the three methods manuals (USEPA, 1993a; USEPA, 1994a; USEPA, 1994b).

1.5 Guidance for the implementation of toxicity tests in the NPDES program is provided in the Technical Support Document for Water Quality-based Toxics Control (USEPA, 1991c).

1.6 The use of any test species or test conditions other than those described in Tables 12-18 in this manual and referenced in Table 1A, 40 CFR 136.3, shall be considered a major modification to the method and subject to application and approval of alternate test procedures under 40 CFR 136.4 and 40 CFR 136.5.

1.7 These methods are restricted to use by, or under the supervision of, analysts experience in the use or conduct of, and interpretation of data from, aquatic toxicity tests. Each analyst must demonstrate the ability to generate acceptable test results with the methods using the procedures described in this methods manual.

1.8 This manual was prepared in the established EMSL-Cincinnati format (USEPA, 1983a).

SECTION 2

TYPES OF TESTS

2.1 The selection of the test type will depend on the NPDES permit requirements, the objectives of the test, the available resources, the requirements of the test organisms, and effluent characteristics such as fluctuations in effluent toxicity.

2.2 Effluent acute toxicity is generally measured using a multi-concentration, or definitive test, consisting of a control and a minimum of five effluent concentrations. The tests are designed to provide dose-response information, expressed as the percent effluent concentration that is lethal to 50% of the test organisms (LC50) within the prescribed period of time (24-96 h), or the highest effluent concentration in which survival is not statistically significantly different from the control.

2.3 Use of pass/fail tests consisting of a single effluent concentration (e.g., the receiving water concentration or RWC) and a control is not recommended. If the NPDES permit has a whole effluent toxicity limit for acute toxicity at the RWC, it is prudent to use that permit limit as the midpoint of a series of five effluent concentrations. This will ensure that there is sufficient information on the dose-response relationship. For example, the effluent concentrations utilized in a test may be: (1) 100% effluent, (2) (RWC + 100)/2, (3) RWC, (4) RWC/2, and (5) RWC/4. More specifically, if the RWC = 50%, appropriate effluent concentrations may be 100%, 75%, 50%, 25%, and 12.5%.

2.4 Receiving (ambient) water toxicity tests commonly employ two treatments, a control and the undiluted receiving water, but may also consist of a series of receiving water dilutions.

2.5 A negative result from an acute toxicity test does not preclude the presence of chronic toxicity. Also, because of the potential temporal variability in the toxicity of effluents, a negative test result with a particular sample does not preclude the possibility that samples collected at some other time might exhibit acute (or chronic) toxicity.

2.6 The frequency with which acute toxicity tests are conducted under a given NPDES permit is determined by the regulatory agency on the basis of factors such as the variability and degree of toxicity of the waste, production schedules, and process changes.

2.7 Tests may be static (static non-renewal or static renewal), or flow-through.

2.7.1 STATIC TESTS

2.7.1.1 Static non-renewal tests - The test organisms are exposed to the same test solution for the duration of the test.

2.7.1.2 Static-renewal tests - The test organisms are exposed to a fresh solution of the same concentration of sample every 24 h or other prescribed interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers.

2.7.2 FLOW-THROUGH TESTS

2.7.2.1 Two types of flow-through tests are in common use: (1) sample is pumped continuously from the sampling point directly to the dilutor system; and (2) grab or composite samples are collected periodically, placed in a tank adjacent to the test laboratory, and pumped continuously from the tank to the dilutor system. The flow-through method employing continuous sampling is the preferred method for on-site tests. Because of the large volume (often 400 L/day) of effluent normally required for flow-through tests, it is generally considered too costly and impractical to conduct these tests off-site at a central laboratory.

2.8 Advantages and disadvantages of the types of tests are as follows:

2.8.1 STATIC NON-RENEWAL TESTS

2.8.1.1 Advantages:

1. Simple and inexpensive.
2. Very cost effective in determining compliance with permit conditions.
3. Limited resources (space, manpower, equipment) required; would permit staff to perform many more tests in the same amount of time.
4. Smaller volume of effluent required than for static renewal or flow-through tests.

2.8.1.2 Disadvantages:

1. Dissolved oxygen (DO) depletion may result from high chemical oxygen demand (COD), biological oxygen demand (BOD), or metabolic wastes.
2. Possible loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Generally less sensitive than static renewal or flow-through tests, because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.8.2 STATIC-RENEWAL, ACUTE TOXICITY TESTS

2.8.2.1 Advantages:

1. Reduced possibility of dissolved oxygen (DO) depletion from high chemical oxygen demand (COD) and/or biological oxygen demand (BOD), or ill effects from metabolic wastes from organisms in the test solutions.
2. Reduced possibility of loss of toxicants through volatilization and/or adsorption to the exposure vessels.
3. Test organisms that rapidly deplete energy reserves are fed when the test solutions are renewed, and are maintained in a healthier state.

2.8.2.2 Disadvantages:

1. Require greater volume of effluent that non-renewal tests.
2. Generally less sensitive than flow-through tests, because the toxic substances may degrade or be adsorbed, thereby reducing the apparent toxicity. Also, there is less chance of detecting slugs of toxic wastes, or other temporal variations in waste properties.

2.8.3 FLOW-THROUGH TESTS

2.8.3.1 Advantages:

1. Provide a more representative evaluation of the acute toxicity of the source, especially if sample is pumped continuously directly from the source and its toxicity varies with time.
2. DO concentrations are more easily maintained in the test chambers.
3. A higher loading factor (biomass) may be used.
4. The possibility of loss of toxicant due to volatilization, adsorption, degradation, and uptake is reduced.

2.8.3.2 Disadvantages:

1. Large volumes of sample and dilution water are required.
2. Test equipment is more complex and expensive, and requires more maintenance and attention.
3. More space is required to conduct tests.
4. Because of the resources required, it would be very difficult to perform multiple or overlapping sequential tests.

SECTION 3

HEALTH AND SAFETY

3.1 GENERAL PRECAUTIONS

3.1.1 Development and maintenance of an effective health and safety program in the laboratory requires an ongoing commitment by laboratory management, and includes (1) the appointment of a laboratory health and safety officer with the responsibility and authority to develop and maintain a safety program, (2) the preparation of a formal, written, health and safety plan, which is provided to each laboratory staff member, (3) an ongoing training program on laboratory safety, and (4) regularly scheduled, documented, safety inspections.

3.1.2 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to lack of oxygen or presence of noxious gases.

3.1.3 Prior to sample collection and laboratory work, personnel must determine that all required safety equipment and materials have been obtained and are in good condition.

3.1.4 Guidelines for the handling and disposal of hazardous materials must be strictly followed.

3.2 SAFETY EQUIPMENT

3.2.1 PERSONAL SAFETY GEAR

3.2.1.1 Personnel must use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes.

3.2.2 LABORATORY SAFETY EQUIPMENT

3.2.2.1 Each laboratory (including mobile laboratories) must be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, and eye fountains.

3.2.2.2 Mobile laboratories should be equipped with a telephone to enable personnel to summon help in case of emergency.

3.3 GENERAL LABORATORY AND FIELD OPERATIONS

3.3.1 Guidance in Material Safety Data Sheets should be followed for reagents and other chemicals purchased from supply houses. Incompatible materials should not be stored together.

3.3.2 Work with effluents must be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see Safety Manuals, Subsection 3.5). Personnel collecting samples and performing toxicity tests should not work alone.

3.3.3 Because the chemical composition of effluents is usually only poorly known, they must be considered as potential health hazards, and exposure to them should be minimized. Fume and canopy hoods over the test areas must be used whenever necessary.

3.3.4 It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

3.3.5 All containers must be adequately labeled to indicate their contents.

3.3.6 Strong acids and volatile organic solvents employed in glassware cleaning must be used in a fume hood or under an exhaust canopy over the work area.

3.3.7 Good housekeeping contributes to safety and reliable results.

3.3.8 Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.3.9 Mobile laboratories must be properly grounded to protect against electrical shock.

3.4 DISEASE PREVENTION

3.4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against hepatitis B, tetanus, typhoid fever, and polio.

3.5 SAFETY MANUALS

3.5.1 For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, check with the permittee and consult general industrial safety manuals, including USEPA (1986) and Walters and Jameson (1984).

3.6 WASTE DISPOSAL

3.6.1 Wastes generated during toxicity testing must be properly handled and disposed of in an appropriate manner. Each testing facility will have its own waste disposal requirements based on local, state, and Federal rules and regulations. It is extremely important that these rules and regulations be known, understood, and complied with by all persons responsible for, or otherwise involved in, performing testing activities. Local fire officials should be notified of any potentially hazardous conditions.

SECTION 4

QUALITY ASSURANCE

4.1 INTRODUCTION

4.1.1 Development and maintenance of a toxicity test laboratory quality assurance (QA) program requires an ongoing commitment by laboratory management, and includes the following: (1) appointment of a laboratory quality assurance officer with the responsibility and authority to develop and maintain a QA program; (2) preparation of a quality assurance plan with data quality objectives; (3) preparation of written descriptions of laboratory standard operating procedures (SOP's) for test organism culturing, toxicity testing, instrument calibration, sample chain-of-custody, laboratory sample tracking system, etc.; and (4) provision of adequate, qualified technical staff and suitable space and equipment to assure reliable data.

4.1.2 QA practices within an aquatic toxicology laboratory must address all activities that affect the quality of the final effluent toxicity data, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition and operation of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation.

4.1.3 Quality control practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance, and general guidance on good laboratory practices related to toxicity testing, see: FDA, 1978; USEPA, 1975; USEPA, 1979a; USEPA, 1980a; USEPA, 1980b; USEPA, 1991b; DeWoskin, 1984; and Taylor, 1987.

4.1.4 Guidance for the evaluation of laboratories performing toxicity tests and laboratory evaluation criteria may be found in USEPA 1991b.

4.2 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

4.2.1 Separate test organism culturing and toxicity testing areas should be provided to avoid possible loss of cultures due to cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemical analysis laboratories or sample storage and preparation areas into organism culturing or toxicity testing areas, and from toxicity test laboratories and sample preparation areas into culture rooms.

4.2.2 Laboratory and toxicity test temperature control equipment must be adequate to maintain recommended test water temperatures. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent (see Section 5, Facilities and Equipment).

4.3 TEST ORGANISMS

4.3.1 The test organisms used in the procedures described in this manual are listed in Section 6, Test Organisms. The organisms should appear healthy, behave normally, feed well, and have low mortality in cultures, during holding, and in test controls. Test organisms should be positively identified to species.

4.4 LABORATORY WATER USED FOR CULTURING AND TEST DILUTION WATER

4.4.1 The quality of water used for test organism culturing and for dilution water used in toxicity tests is extremely important. Water for these two uses should come from the same source. The dilution water used in effluent toxicity tests will depend in part on the objectives of the study and logistical constraints, as discussed in detail in Section 7, Dilution Water. The dilution water used for internal quality assurance tests with organisms, food, and reference toxicants should be the water routinely used with success in the laboratory. Types of water are discussed in Section

5, Facilities and Equipment. Water used for culturing and test dilution should be analyzed for toxic metals and organics at least annually or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction or growth. The concentration of the metals, Al, As, Cr, Co, Cu, Fe, Pb, Ni, and Zn, expressed as total metal, should not exceed 1 µg/L each, and Cd, Hg, and Ag, expressed as total metal, should not exceed 100 ng/L each. Total organochlorine pesticides plus PCBs should be less than 50 ng/L (APHA, 1992). Pesticide concentrations should not exceed USEPA's Ambient Water Quality chronic criteria values where available.

4.5 **EFFLUENT SAMPLING AND SAMPLE HANDLING**

4.5.1 Sample holding times and temperatures must conform to conditions described in Section 8, Effluent and Receiving Water Sampling and Sample Handling.

4.6 **TEST CONDITIONS**

4.6.1 The temperature of test solutions must be measured by placing the thermometer or probe directly into the test solutions, or by placing the thermometer in equivalent volumes of water in surrogate vessels positioned at appropriate locations among the test vessels. Temperature should be recorded continuously in at least one vessel during the duration of each test. Test solution temperatures should be maintained within the limits specified for each test. DO concentration and pH in test chambers should be checked daily throughout the test period, as prescribed in Section 9, Acute Toxicity Test Procedures.

4.7 **QUALITY OF TEST ORGANISMS**

4.7.1 The health of test organisms is primarily assessed by the performance (survival, growth, and/or reproduction) of organisms in control treatments of individual tests. The health and sensitivity of test organisms is also assessed by reference toxicant testing. In addition to documenting the sensitivity and health of test organisms, reference toxicant testing is used to initially demonstrate acceptable laboratory performance (Subsection 4.14) and to document ongoing laboratory performance (Subsection 4.15).

4.7.2 Regardless of the source of test organisms (in-house cultures or purchased from external suppliers), the testing laboratory must perform at least one acceptable reference toxicant test per month for each toxicity test method conducted in that month (Subsection 4.15). If a test method is conducted only monthly, or less frequently, a reference toxicant test must be performed concurrently with each effluent toxicity test.

4.7.3 When acute or short-term chronic toxicity tests are performed with effluents or receiving waters using test organisms obtained from outside the test laboratory, concurrent toxicity tests of the same type must be preformed with a reference toxicant, unless the test organism supplier provides control chart data from at least the last five monthly acute toxicity tests using the same reference toxicant and test conditions.

4.7.4 The supplier should also certify the species identification of the test organisms, and provide the taxonomic reference (citation and page) or name(s) of the taxonomic expert(s) consulted.

4.7.5 If a routine reference toxicant test fails to meet test acceptability criteria, then the reference toxicant test must be immediately repeated.

4.8 **FOOD QUALITY**

4.8.1 The nutritional quality of the food used in culturing and testing fish and invertebrates is an important factor in the quality of the toxicity test data. This is especially true for the unsaturated fatty acid content of brine shrimp nauplii, *Artemia*. Suitable trout chow, *Artemia*, and other foods must be obtained as described in this manual.

4.8.2 Problems with the nutritional suitability of the food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. If a batch of food is suspected to be defective, the performance of organisms fed with the new food can be compared with the performance of organisms fed with a food of known quality in side-by-side tests. If the food is used for culturing, its suitability should be determined using a short-term chronic test which will determine the effect of food quality on growth or reproduction of each of the relevant test species in culture, using four replicates with each food source. Where applicable, foods used only in acute toxicity tests can be compared with a food of known quality in side-by-side, multi-concentration acute tests, using the reference toxicant regularly employed in the laboratory QA program.

4.8.3 New batches of food used in culturing and testing should be analyzed for toxic organics and metals or whenever difficulty is encountered in meeting minimum test acceptability criteria for control survival and reproduction or growth. If the concentration of total organochlorine pesticides exceeds 0.l5 µg/g wet weight, or the concentration of the total organochlorine pesticides plus PCBs exceeds 0.30 µg/g wet weight, or toxic metals (Al, As, Cr, Co, Cu, Pb, Ni, Zn, expressed as total metal) exceed 20 µg/g wet weight, the food should not be used (for analytical methods see AOAC, 1990 and USDA, 1989). For foods (e.g., such as YCT) which are used to culture and test organisms, the quality of food should meet the requirements for the laboratory water used for culturing and test dilution water as described in Section 4.4 above.

4.9 ACCEPTABILITY OF ACUTE TOXICITY TEST RESULTS

4.9.1 For the test results to be acceptable, control survival must equal or exceed 90%.

4.9.2 An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test condition summaries). The acceptability of the test will depend on the experience and professional judgment of the laboratory analyst and the reviewing staff of the regulatory authority. Any deviation from test specifications must be noted when reporting data from a test.

4.10 ANALYTICAL METHODS

4.10.1 Routine chemical and physical analyses for culture and dilution water, food, and test solutions, must include established quality assurance practices outlined in Agency methods manuals (USEPA, 1979a; USEPA, 1993b).

4.10.2 Reagent containers should be dated when received from the supplier, and the shelf life should not be exceeded. Also, working solutions should be dated when prepared, and the recommended shelf life should be observed.

4.11 CALIBRATION AND STANDARDIZATION

4.11.1 Instruments used for routine measurements of chemical and physical parameters such as pH, DO, temperature, conductivity, salinity, alkalinity, and hardness must be calibrated and standardized prior to use each day according to the instrument manufacturer's procedures as indicated in the general section on quality assurance (see EPA Methods 150.1, 360.1, 170.1, and 120.1; USEPA, 1979b). Calibration data are recorded in a permanent log.

4.11.2 Wet chemical methods used to measure hardness, alkalinity, and total residual chlorine must be standardized prior to use each day according to the procedures for those specific EPA methods (see EPA Methods 130.2 and 310.1; USEPA 1979b).

4.12 **REPLICATION AND TEST SENSITIVITY**

4.12.1 The sensitivity of toxicity tests will depend in part on the number of replicates per concentration, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data.

4.13 **VARIABILITY IN TOXICITY TEST RESULTS**

4.13.1 Factors which can affect test success and precision include: the experience and skill of the laboratory analyst; test organism age, condition, and sensitivity; dilution water quality; temperature control; and the quality and quantity of food provided. The results will depend upon the species used and the strain or source of the test organisms, and test conditions such as temperature, DO, food, and water quality. The repeatability or precision of toxicity tests is also a function of the number of test organisms used at each toxicant concentration. Jensen (1972) discussed the relationship between sample size (numbers of fish) and the standard error of the test, and considered 20 fish per concentration as optimum for Probit Analysis.

4.13.2 Test precision can be estimated by using the same strain of organisms under the same test conditions, and employing a known toxicant, such as a reference toxicant. The single-laboratory (intra-laboratory) and multi-laboratory (inter-laboratory) precision of acute toxicity tests with several common test species and reference toxicants are listed in Tables 1-4. Intra- and inter-laboratory precision are described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation, or CV) of the calculated endpoints from the replicated tests.

4.13.3 Intra-laboratory precision data from 268 acute toxicity tests with four species and five reference toxicants are listed in Tables 1 and 2. The precision, expressed as CV%, ranged from 3% to 86%. More recent CV values reported by Jop et al. (1986), Dorn and Rogers (1989), Hall et al. (1989), and Cowgill et al. (1990), fell in a somewhat lower range (8% to 41%).

4.13.4 Inter-laboratory precision of acute toxicity tests from 253 reference toxicant tests with seven species, listed in Tables 2, 3, 4, and 5 (expressed as CV% for LC50s), ranged from 11% to 167%. Table 6 shows interlaboratory precision data from a study of acute toxicity test methods using reference toxicant, effluent, and receiving water sample types (USEPA, 2001a; USEPA, 2001b). Averaged across sample types, total interlaboratory precision (expressed as CV% for LC50s) ranged from 13% to 38.5% for the acute methods.

4.13.5 No clear pattern of differences were noted in the intra- or inter-laboratory test precision with the species listed, although the test results with some toxicants, such as cadmium, appear to more variable than those with other reference toxicants.

4.13.6 Additional information on toxicity test precision is provided in the Technical Support Document for Water Quality-Based Toxics Control (see pp. 2-4, and 11-15; USEPA, 1991c).

TABLE 1. INTRA-LABORATORY PRECISION OF LC50S FROM STATIC ACUTE TOXICITY TESTS WITH AQUATIC ORGANISMS USING REFERENCE TOXICANTS[1]

		REFERENCE TOXICANT [2]								
		SDS			NAPCP			CD		
TEST ORGANISM		N	LC50	CV (%)	N	LC50	CV (%)	N	LC50	CV (%)
Pimephales promelas	(96 h, 21°C)[3]	9	8.6	20	12	0.14	40	9	0.15	120
Daphnia magna	(24 h, 20°C)[4]	8	20.9	28	10	0.69	14	11	0.121	49
Daphnia magna	(24 h, 26°C)[4]	10	12.9	48	9	0.67	25	9	0.026	77
Daphnia magna	(48 h, 20°C)[4]	10	13.5	29	10	0.42	21	9	0.038	58
Daphnia magna	(48 h, 26°C)[4]	9	10.8	33	9	0.48	23	8	0.009	35
Daphnia pulex	(24 h, 20°C)[4]	9	18.4	23	9	0.64	15	5	0.147	30
Daphnia pulex	(24 h, 26°C)[4]	10	13.9	25	9	0.62	25	10	0.063	45
Daphnia pulex	(48 h, 20°C)[4]	10	12.6	32	9	0.48	16	10	0.042	45
Daphnia pulex	(48 h, 26°C)[4]	9	10.2	36	8	0.47	32	6	0.006	14
Mysidopsis bahia	(96 h, 25°C)[5]							13	0.346	9

[1] Precision expressed as percent coefficient of variation, where CV% = (standard deviation X 100)/mean.
[2] SDS = Sodium dodecyl (lauryl) sulfate; NAPCP = Sodium pentachlorophenate; CD = Cadmium; N = Number of tests; toxicant concentration in mg/L.
[3] *Pimephales promelas* tests were performed in soft, synthetic freshwater; total hardness, 40-48 mg/L as $CaCO_3$, by J. Dryer, Aquatic Biology Section, EMSL-Cincinnati.
[4] *Daphnia* data from Lewis and Horning, 1991. Tests with *D. magna* used hard reconstituted water (total hardness, 180-200 mg/L as $CaCO_3$); tests with *D. pulex* used moderately-hard reconstituted water (total hardness, 80-100 mg/L as $CaCO_3$).
[5] Mysid tests were performed in 25 ppt salinity, natural seawater. Data were provided by Steve Ward, Environmental Services Division, U.S. Environmental Protection Agency, Edison, New Jersey. Personal communication, November 14, 1990.

TABLE 2. INTRA- AND INTER-LABORATORY PRECISION OF ACUTE TOXICITY TESTS WITH *DAPHNIA MAGNA*, USING A STANDARD EFFLUENT[1,2]

LABORATORY	INTER-LABORATORY PRECISION: LC50s FROM REPLICATE TESTS 24 H	48 H	INTRA-LABORATORY PRECISION[3]
INDUSTRY			
1	14.4	4.2	
	11.4	4.9	---
2	13.9	6.8	
	16.6	6.1	
	13.7	6.1	6.4
3	11.7	3.5	
	17.4	7.1	---
GOVERNMENT			
1	14.0	4.4	
	10.0	4.4	
	10.8	4.1	4.0
2	13.2	4.5	
	14.1	4.5	---
3	11.6	4.2	---
COMMERCIAL			
1	20.1	4.9	
	20.1	4.7	---
2	8.9	3.7	
	12.3	5.6	---
3	14.8	9.0	
	25.4	9.1	
	26.4	8.6	3.0
N	20	20	3
MEAN	15.0	5.52	4.47
SD	4.75	1.75	1.75
CV%	31.6	31.6	39.1

1 From Table 2, p. 191, Grothe and Kimerle, 1985. Tests performed at 20°C ±2°C; dilution water hardness, 100mg/L as $CaCO_3$; dilution water alkalinity, 76 mg/L as $CaCO_3$; effluent hardness, approx. 1000 mg/L as $CaCO_3$; effluent alkalinity, 310 mg/L as $CaCO_3$; effluent dilutions - 56%, 32%, 18%, 10%, 5.6%, 3.1%, 1.7%.

2 LC50 expressed in percent effluent.

3 Intra-laboratory precision expressed as the weighted mean CV(%).

TABLE 3. INTER-LABORATORY PRECISION OF ACUTE TOXICITY TESTS WITH AQUATIC ORGANISMS, USING REFERENCE TOXICANTS[1]

	REFERENCE TOXICANT					
	SILVER			ENDOSULFAN		
TEST ORGANISM	N	LC50	CV (%)	N	LC50	CV (%)
1. *Pimephales promelas* (96 h, 22°C)						
96-h static test (Meas)	10	14.0	53	12	2.03	38
96-h flow-through test (Meas)	9	7.49	40	12	0.96	46
2. *Oncorhyncus mykiss* (96 h, 12°C)						
96-h static test (Meas)	10	34.5	88	12	1.15	50
96-h flow-through test (Meas)	9	11.5	33	12	0.40	42
3. *Daphnia magna* (48 h, 20°C)						
48-h static (Meas)	12	10.6	166	11	328	51
4. *Mysidopsis bahia* (96 h, 22°C)						
96-h static test (Nom)	6	210	27	5	0.84	62
96-h flow-through test (Nom)	6	251	22	6	1.02	58
96-h flow-through test (Meas)	6	192	58	5	0.94	167
5. *Cyprinodon variegatus* (96 h, 22°C)						
96-h static test (Nom)	4	1122	35	6	2.41	37
96-h flow-through test (Nom)	5	1573	50	6	1.69	46
96-h flow-through test (Meas)	5	1216	50	6	0.81	46

[1] Data for *Pimephales promelas* (fathead minnow), *Oncorhyncus mykiss* (rainbow trout), and *Daphnia magna* were taken from USEPA, 1983b.

Data for, *Mysidopsis bahia*, and *Cyprinodon variegatus* (sheepshead minnow) were taken from USEPA, 1981. Six laboratories participated in each study. Test salinity was 28‰.

LC50s expressed in µg/L.

In the studies with the freshwater organisms, the water hardness for five of the six laboratories ranged between 36 and 75 mg/L. However, the water hardness for the sixth laboratory was 255 mg/L, resulting in LC50 values for silver more than an order of magnitude larger than for the other five. These values were rejected in calculating the CV%. The mean weights of test fish were from 0.05-0.26 g for fathead minnows, and 0.22-1.32 g for rainbow trout. *Daphnia* were ≤24-h old.

In studies with the marine organisms, only one LC50 (presumably the combined LC50 from duplicate tests) was reported for each toxicity test. LC50s for flow-through tests with *Mysidopsis bahia* and Cyprinodon *variegatus* were calculated two different ways -- (1) on the basis of the nominal toxicant concentrations (Nom), and (2) on the basis of measured (Meas) toxicant concentrations. Test organism age was ≤2 days for *Mysidopsis bahia*, and 28 days for *Cyprinodon variegatus*. The salinity of test solutions was 28‰.

N, the total number of LC50 values used in calculating the CV(%) varied with organism and toxicant because some data were rejected due to water hardness, lack of concentration measurements, and/or because some of the LC50s were not calculable.

[2] CV% = Percent coefficient of variation = (standard deviation x 100)/mean.

TABLE 4. INTER-LABORATORY STUDY OF ACUTE TOXICITY TEST PRECISION, 1990: SUMMARY OF RESPONSES USING KCL AS THE REFERENCE TOXICANT[1]

			TEST PRECISION (CV%)[2]								
			GRAPH[3] METHOD			STAT[4] METHOD			TOTAL[5]		
TEST TYPE		NO. LABS SUBMITTING VALID DATA	N	LC50	CV%	N	LC50	CV%	N	LC50	CV%
Pimephales promelas	(96 h, 22°C)[6]	17	6	944	28.8	13	832	27.8	17	864	29.6
Pimephales promelas	(24 h, 25°C)[7]	6	6	832	11.5	6	832	11.5	–	–	–
Ceriodaphnia dubia	(48 h, 25°C)[7]	11	11	256	53.1	11	264	48.5	–	–	–
Mysidopsis bahia	(96 h, 22°C)[8]	14	7	292	32.9	11	250	36.0	14	268	37.3

1 Interlaboratory study of toxicity test precision conducted in 1990 by the Environmental Monitoring Systems Laboratory - Cincinnati, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268, in cooperation with the states of New Jersey and North Carolina, and the Office of Water Enforcement and Permits, U.S. Environmental Protection Agency, Washington, DC.

2 Percent coefficient of variation = (standard deviation X 100)/mean. Calculated for LC50 from acute tests. LC50s expressed as mg/L KCl added to the dilution water.

3 LC50 estimated by the Graphical Method.

4 LC50 estimated by Probit, Litchfield-Wilcoxon, or Trimmed Spearman-Karber method.

5 LC50 usually reported for only one method of analysis for each test. Where more than one LC50 was reported for a test, the lowest value was used to calculate the statistics for "Total."

6 Data from the New Jersey Department of Environmental Protection: static daily-renewal tests, using moderately-hard synthetic freshwater.

7 Data from North Carolina certified laboratories: static non-renewal tests, using moderately-hard reconstituted freshwater.

8 Data from the New Jersey Department of Environmental Protection: static daily-renewal tests, using 25 ppt salinity, FORTY FATHOMS® synthetic seawater.

TABLE 5. NATIONAL INTERLABORATORY STUDY OF ACUTE TOXICITY TEST PRECISION, 1991: SUMMARY OF RESPONSES USING REFERENCE TOXICANTS[1]

Test Type		No. Labs Submitting Data	LC50	CV%[2]
Pimephales promelas	(48 h, 25°C)[3]	203	896[4]	28.6
Ceriodaphnia dubia	(48 h, 25°C)[3]	171	432[4]	39.8
Mysidopsis bahia	(48 h, 25°C)[5]	61	532[4]	30.1
Menidia beryllina	(48 h, 25°C)[5]	39	164[6]	42.2

1 From a national study of interlaboratory precision of toxicity test data performed in 1991 by the Environmental Monitoring Systems Laboratory - Cincinnati, U.S. Environmental Protection Agency, Cincinnati, OH 45268. Participants included Federal, state, and private laboratories engaged in NPDES permit compliance monitoring. LC50s were estimated by the graphical or Spearman-Karber method.
2 Percent coefficient of variation = (standard deviation X 100)/mean.
3 Static non-renewal tests, using moderately-hard synthetic freshwater (total hardness = 80-100 mg/L as $CaCO_3$).
4 Expressed as mg KCl added per liter of dilution water.
5 Static non-renewal tests, using 30 ppt modified GP2 artificial seawater.
6 Expressed as μg Cu^{++} added per liter of dilution water.

TABLE 6. NATIONAL INTERLABORATORY STUDY OF ACUTE TOXICITY TEST PRECISION, 2000: PRECISION OF LC50 POINT ESTIMATES FOR REFERENCE TOXICANT, EFFLUENT, AND RECEIVING WATER SAMPLE TYPES[1].

Method	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
Pimephales promelas	KCl	7.62	19.7	21.1
	Municipal effluent	10.3	19.2	21.8
	Receiving water	-	-	17.2
	Average	8.96	19.4	20.0
Ceriodaphnia dubia	KCl	14.6	15.2	21.1
	Municipal effluent	9.68	32.8	34.2
	Receiving water	-	-	31.8
	Average	12.1	24.0	29.0
Cyprinodon variegatus	KCl	-	-	26.0
	Municipal effluent	-	-	19.4
	Receiving water	-	-	32.5
	Average	-	-	26.0
Menidia beryllina	$CuSO_4$[6]	-	-	-
	Industrial effluent	9.91	49.7	50.7
	Receiving water	-	-	26.3
	Average	9.91	49.7	38.5
Holmesimysis costata[7]	Zn (48 h test)	19	-	
	Zn (96 h test)	23	-	
	Zn (interlaboratory trial 1)	-	-	24
	Zn (interlaboratory trial 2)	-	-	1
	Average	21		13

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type. The study design also did not provide within-laboratory replication for the *Cyprinodon variegatus* Acute Method.

[3] The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.
[4] The between-laboratory component of variability for duplicate samples tested at different laboratories.
[5] The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.
[6] Precision estimates were not calculated for the reference toxicant sample type since the majority of results for this sample type were outside of the test concentration range (ie., >100).
[7] *Holmesimysis costata* Acute Test data were from Martin *et al.* (1989). Zn was tested in two intralaboratory trials and in two interlaboratory trials. Data from this study was only reported to two significant figures.

4.14 **DEMONSTRATING ACCEPTABLE LABORATORY PERFORMANCE**

4.14.1 It is a laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with reference toxicants before it performs toxicity tests with effluents for permit compliance purposes. To meet this requirement, the intra-laboratory precision, expressed as percent coefficient of variation (CV%), of each type of test to be used in a laboratory should be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (i.e., the same test duration, type of dilution water, age of test organisms, feeding, etc.), and same data analysis methods. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations.

4.15 **DOCUMENTING ONGOING LABORATORY PERFORMANCE**

4.15.1 Satisfactory laboratory performance is demonstrated by performing at least one acceptable test per month with a reference toxicant for each toxicity test method conducted in the laboratory during that month. For a given test method, successive tests must be performed with the same reference toxicant, at the same concentrations, in the same dilution water, using the same data analysis methods. Precision may vary with the test species, reference toxicant, and type of test. Each laboratory's reference toxicity data will reflect conditions unique to that facility, including dilution water, culturing, and other variables; however, each laboratory's reference toxicity results should reflect good repeatability.

4.15.2 A control chart should be prepared for each combination of reference toxicant, test species, test condition, and endpoint. Toxicity endpoints from five or six tests are adequate for establishing the control charts. In this technique, a running plot is maintained for the toxicity values (X_i) from successive tests with a given reference toxicant (Figure 1), and endpoints (LC50s) are examined to determine if they are within prescribed limits. The types of control charts illustrated (see USEPA, 1979a) are used to evaluate the cumulative trend of results from a series of samples, thus reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. The mean ($\bar{x}$) and upper and lower control limits (±2S) are re-calculated with each successive test result. After two years of data collection, or a minimum of 20 data points, the control chart should be maintained using only the 20 most recent data points.

4.15.3 Laboratories should compare the calculated CV (i.e., standard deviation / mean) of the LC50 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-3 in USEPA, 2000b). If the calculated CV exceeds the 75th percentile of CVs reported nationally, the laboratory should use the 75th and 90th percentiles to calculate warning and control limits, respectively, and the laboratory should investigate options for reducing variability.

4.15.4 The outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, are readily identified. At the $P_{0.05}$ probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone. If more than one out of 20 reference toxicant tests fall outside the control limits, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. In those instances when the laboratory can document the cause for the outlier (e.g., operator error, culture health or test system failure), the outlier should be excluded from the future calculations of the control limits. If two or more consecutive tests do not fall within the control limits, the results must be explained and the reference toxicant test must be immediately repeated. Actions taken to correct the problem must be reported.

4.15.5 If the toxicity value from a given test with the reference toxicant falls well outside the expected range for the test organisms when using the standard dilution water, the laboratory should investigate sources of variability, take corrective actions to reduce identified sources of variability, and perform an additional reference toxicant test during the same month. Performance should improve with experience, and the control limits for point estimates should gradually narrow. However, control limits of ±2S, by definition, will be exceeded 5% of the time, regardless of how well a laboratory performs. Highly proficient laboratories which develop a very narrow control limit may be unfairly penalized if a test which falls just outside the control limits is rejected *de facto*. For this reason, the width of the control limits should be considered in determining whether or not a reference toxicant test result falls “well” outside the expected range. The width of the control limits may be evaluated by comparing the calculated CV (i.e., standard deviation / mean) of the LC50 for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing (Table 3-3 in USEPA, 2000b). In determining whether or not a reference toxicant test result falls “well” outside the expected range, the result also may be compared with upper and lower bounds for ±3S, as any result outside these control limits would be expected to occur by chance only 1 out of 100 tests (Environment Canada, 1990). When a result from a reference toxicant test is outside the 99% confidence intervals, the laboratory must conduct an immediate investigation to assess the possible causes for the outlier.

4.15.6 Reference toxicant test results should not be used as a *de facto* criterion for rejection of individual effluent or receiving water tests. Reference toxicant testing is used for evaluating the health and sensitivity of organisms over time and for documenting initial and ongoing laboratory performance. While reference toxicant test results should not be used as a *de facto* criterion for test rejection, effluent and receiving water test results should be reviewed and interpreted in the light of reference toxicant test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increased test organism sensitivity or toward decreased test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test.

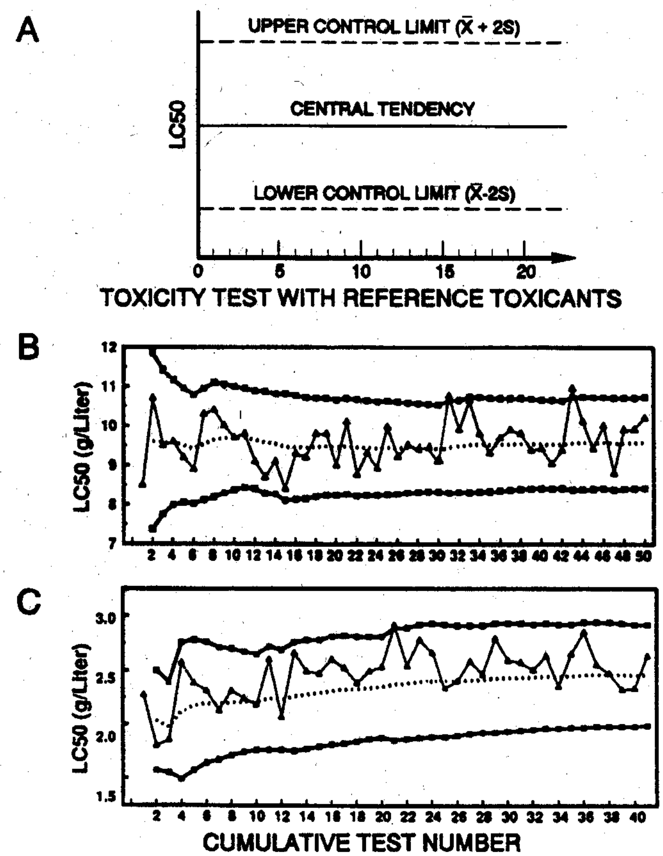

Figure 1. Control (cusum) charts: A, General case; B and C, 48-h acute tests with sodium chloride. (B) Fathead minnow (*Pimephales promelas*), and (C) *Ceriodaphnia dubia*, with the individual LC50s (Triangles), cumulative LC50 means (dotted line), and upper and lower control limits of two standard deviations (squares). (Provided by the Environmental Services Division, U.S. Environmental Protection Agency, Kansas City, KS).

4.16 **REFERENCE TOXICANTS**

4.16.1 Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride ($CdCl_2$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$), are suitable for use in the NPDES and other Agency programs requiring aquatic toxicity tests. EMSL-Cincinnati hopes to release EPA-certified solutions of cadmium and copper, with accompanying toxicity data for the recommended test species, for use as reference toxicants through cooperative research and development agreements with commercial suppliers, and will continue to develop additional reference toxicants for future release. Standard reference materials can be obtained from commercial supply houses, or can be prepared inhouse using reagent grade chemicals. The regulatory agency should be consulted before reference toxicant(s) are selected and used.

4.17 **RECORD KEEPING**

4.17.1 Proper record keeping is important. A complete file should be maintained for each individual toxicity test or group of tests on closely related samples. This file should contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the toxicity test(s); chemical analysis data on the sample(s); detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions employed; and results of reference toxicant tests. Laboratory data should be recorded on a real-time basis to prevent the loss of information or inadvertent introduction of errors into the record. Original data sheets should be signed and dated by the laboratory personnel performing the tests.

4.17.2 The regulatory authority should retain records pertaining to discharge permits. Permittees are required to retain records pertaining to permit applications and compliance for a minimum of 3 years [40 CFR 122.41(j)(2)].

SECTION 5

FACILITIES AND EQUIPMENT

5.1 GENERAL REQUIREMENTS

5.1.1 Effluent toxicity tests may be performed in a fixed or mobile laboratory. Facilities should include equipment for rearing and/or holding organisms.

5.1.2 The facilities must be well ventilated and free of toxic fumes. Sample preparation, culturing, and toxicity testing areas should be separated to avoid cross contamination of cultures or toxicity test solutions with toxic fumes. Laboratory ventilation systems should be checked to ensure that return air from chemistry laboratories and/or sample handling areas is not circulated to test organism culture rooms or toxicity test rooms, or that air from toxicity test rooms does not contaminate culture areas. Air pressure differentials between such rooms should not result in a net flow of potentially contaminated air to sensitive areas through open or loosely-fitting doors.

5.1.3 Control of test solution temperature can best be achieved using circulating water baths, heat exchangers, or environmental chambers. Photoperiod can be controlled using automatic timers in the laboratory or environmental chambers.

5.1.4 Water used for rearing, holding, and testing organisms may be reconstituted synthetic water, ground water, surface water, or dechlorinated tap water. Dechlorination can be accomplished by carbon filtration, laboratory water conditioning units, or the use of sodium thiosulfate. After dechlorination, total residual chlorine should be non-detectable. Sodium thiosulfate may be toxic to the test organisms, and if used for dechlorination, paired controls with and without sodium thiosulfate should be incorporated in effluent toxicity tests. Use of 3.6 mg (anhydrous) sodium thiosulfate/L will reduce l.0 mg chlorine/L. After dechlorination, total residual chlorine should be non-detectable.

5.1.4.1 A good quality, laboratory grade deionized water, providing a resistance of 18 megaohm-cm, must be available in the laboratory and in sufficient quantity for laboratory needs. Deionized water may be obtained from MILLIPORE®, Milli-Q®, MILLIPORE QPAK™$_2$ or equivalent system. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade water deionizer with preconditioned water from a CULLIGAN®, CONTINENTAL®, or equivalent, mixed-bed water treatment system.

5.1.5 Air used for aeration must be free of oil and fumes. Oil-free air pumps should be used where possible. Particulates can be removed from the air using BALSTON® Grade BX or equivalent filters (Balston, Inc., Lexington, MA), and oil and other organic vapors can be removed using activated carbon filters (BALSTON®, C-1 filter, or equivalent).

5.1.6 During rearing, holding, and testing, test organisms should be shielded from external disturbances such as rapidly changing light conditions (especially salmonids) and pedestrian traffic.

5.1.7 Materials used for exposure chambers, tubing, etc., that come in contact with the effluent and dilution water should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON®) should be used whenever possible to minimize sorption and leaching of toxic substances, and may be reused after cleaning. Containers made of plastics, such as polyethylene, polypropylene, polyvinyl chloride, TYGON®, etc., may be used to ship, store, and transfer effluents and receiving waters, but they should not be reused unless absolutely necessary, because they could carry over adsorbed toxicants from one test to another. However, these containers may be repeatedly reused for storing uncontaminated waters such as deionized or laboratory-prepared dilution waters and receiving waters. Glass or disposable polystyrene containers can be used as test chambers. The use of large ($\geq$20 L) glass carboys is discouraged for safety reasons.

5.1.8 New plastic products should be tested for toxicity before general use by exposing organisms to them under ordinary test conditions.

5.1.9 Equipment which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below. Fiberglass, in addition to the previously mentioned materials, can be used for holding and dilution water storage tanks, and in the water delivery system. All material should be flushed or rinsed thoroughly with dilution water before using in the test.

5.1.10 Copper, galvanized material, rubber, brass, and lead must not come in contact with holding or dilution water, or with effluent samples and test solutions. Some materials, such as neoprene rubber (commonly used for stoppers), may be toxic and should be tested before use.

5.1.11 Silicone adhesive used to construct glass test chambers absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

5.2 CLEANING TEST CHAMBERS AND LABORATORY APPARATUS

5.2.1 New plasticware used for effluent or dilution water collection or organism test chambers does not require thorough cleaning before use. It is sufficient to rinse new sample containers once with sample dilution water before use. New glassware must be soaked overnight in 10% acid (see below) and rinsed well in deionized water and dilution water.

5.2.2 All non-disposable sample containers, test vessels, tanks, and other equipment that has come in contact with effluent must be washed after use in the manner described below to remove surface contaminants as described below:

1. Soak 15 min in tap water, and scrub with detergent, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Carefully rinse once with fresh, dilute (10%, V:V) hydrochloric or nitric acid to remove scale, metals, and bases. To prepare a 10% solution of acid, add 10 mL of concentrated acid to 90 mL of deionized water.
4. Rinse twice with deionized water.
5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).
6. Rinse three times with deionized water.

5.2.3 All test chambers and equipment should be thoroughly rinsed with the dilution water immediately prior to use in each test.

5.3 APPARATUS AND EQUIPMENT FOR CULTURING AND TOXICITY TESTS

5.3.1 Culture units -- see Appendix. It is preferable to obtain test organisms from in-house culture units. If it is not feasible to maintain cultures in-house, test organisms can be obtained from commercial sources, and should be shipped to the laboratory in well oxygenated water in insulated containers to minimize excursions in water temperature during shipment. The temperature of the water in the shipping containers should be measured on arrival, to determine if the organisms were subjected to obvious undue thermal stress.

5.3.2 Samplers -- automatic samplers, preferably with sample cooling capability, that can collect a 24-h composite sample of 2 L or more.

5.3.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

5.3.4 Environmental chamber or equivalent facility with temperature control (20°C or 25°C)

5.3.5 Water purification system -- MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$, or equivalent. Depending on the quantity of high grade water needed, a first-stage pre-conditioner deionizer, such as a Culligan® or Continental® System, or equivalent, may be needed to provide feed water to the high-purity system.

5.3.6 Balance -- analytical, capable of accurately weighing to 0.0001 g.

5.3.7 Reference weights, Class S -- for documenting the performance of the analytical balance(s). The balance(s) should be checked with reference weights which are at the upper and lower ends of the range of the weighings made when the balance is used. A balance should be checked at the beginning of each series of weighings, periodically (such as every tenth weight) during a long series of weighings, and after the last weight of a series is taken.

5.3.8 Test chambers -- borosilicate glass or non-toxic disposable plastic test chambers are suitable. Test chamber volumes are indicated in the method summaries. To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic, 6 mm (¼ in) thick.

5.3.9 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

5.3.10 Volumetric pipets -- Class A, 1-100 mL.

5.3.11 Serological pipets -- 1-10 mL, graduated.

5.3.12 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

5.3.13 Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring test organisms.

5.3.14 Wash bottles -- for rinsing small glassware and instrument electrodes and probes.

5.3.15 Glass or electronic thermometers -- for measuring water temperature.

5.3.16 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

5.3.17 National Bureau of Standards Certified thermometer (see USEPA Method 170.1; USEPA 1979b).

5.3.18 pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

5.3.19 Refractometer -- for measuring effluent, receiving, and test solution salinity.

5.3.20 Amperometric titrator -- for measuring total residual chlorine.

5.4 REAGENTS AND CONSUMABLE MATERIALS

5.4.1 Reagent water -- defined as MILLIPORE® MILLI-Q®, MILLIPORE® QPAK™$_2$ or equivalent water (see Subsection 5.3.5 above).

5.4.2 Effluent, dilution water, and receiving water -- see Section 7, Dilution Water, and Section 8, Effluent and Receiving Water Sampling and Sample Handling.

5.4.3 Reagents for hardness and alkalinity tests (see USEPA Methods 130.2 and 310.1; USEPA l979b).

5.4.4   Standard pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for instrument calibration (see USEPA Method 150.1; USEPA 1979b).

5.4.5   Specific conductivity and salinity standards (see USEPA Method 120.1; USEPA 1979b).

5.4.6   Laboratory quality control check samples and standards for the above chemistry methods.

5.4.7   Reference toxicant solutions (see Section 4, Quality Assurance).

5.4.8   Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1; USEPA 1979b), or reagents for modified Winkler analysis.

5.4.9   Sources of Food for Cultures and Toxicity Tests.

5.4.9.1   All food should be tested for nutritional suitability, and chemically analyzed for organic chlorine, PCBs, and toxic metals (see Section 4, Quality Assurance).

5.4.9.2   Brine Shrimp (*Artemia*) -- see Appendix A.

1. Brine Shrimp (*Artemia*) Cysts.

   There are many commercial sources of brine shrimp cysts. The quality of the cysts may vary from one batch to another, and the cysts in each new batch (can or lot) should be evaluated for nutritional suitability and chemical contamination. The nutritional suitability (see Leger et al., 1985, 1986) of each new batch is checked against known suitable reference cysts by performing a side-by-side growth and/or reproduction tests using the "new" and "reference" cysts. If the results of tests for nutritional suitability or chemical contamination do not meet standards, the *Artemia* should not be used.

2. Frozen Adult Brine Shrimp

   Frozen adult brine shrimp are available from pet stores and other commercial sources.

5.4.9.3   Trout Chow

Starter or No. 1 pellets, prepared according to current U.S. Fish and Wildlife Service specifications, are available from commercial sources. (The flake food, TETRAMIN® or BIORIL®, can be used regularly as a substitute for trout chow in preparing food for daphnids, and can be used as a short-term substitute for trout chow in feeding fathead minnows.)

5.4.9.4   Dried, Powdered Leaves (CEROPHYLL®)

Dried, powdered, cereal leaves (e.g., CEROPHYLL® or equivalent) are available from commercial suppliers. Dried, powdered, alfalfa leaves obtained from health food stores have been found to be a satisfactory substitute for cereal leaves.

5.4.9.5   Yeast

Packaged dry yeast, such as Fleischmann's, or equivalent, can be purchased at the local grocery store.

5.4.9.6   Flake Fish Food

The flake foods, TETRAMIN® and BIORIL®, are available at most pet supply shops.

5.5 TEST ORGANISMS

5.5.1 Test organisms are obtained from inhouse cultures or commercial suppliers (see Section 6, Test Organisms).

SECTION 6

TEST ORGANISMS

6.1 TEST SPECIES

6.1.1 The species used in characterizing the acute toxicity of effluents and/or receiving waters will depend on the requirements of the regulatory authority and the objectives of the test. It is essential that good quality test organisms be readily available throughout the year from inhouse or commercial sources to meet NPDES monitoring requirements. The organisms used in toxicity tests must be identified to species. If there is any doubt as to the identity of the test organisms, representative specimens should be sent to a taxonomic expert to confirm the identification.

6.1.2 Toxicity test conditions and culture methods are provided in this manual for the following principal test organisms:

Freshwater Organisms:

1. *Ceriodaphnia dubia* (daphnid) (Table 12).
2. *Daphnia pulex* and *D. magna* (daphnids) (Table 13).
3. *Pimephales promelas* (fathead minnow) (Table 14).
4. *Oncorhynchus mykiss* (rainbow trout) and *Salvelinus fontinalis* (brook trout) (Table 15).

Estuarine and Marine Organisms:

1. *Mysidopsis bahia* (mysid) (Table 16).[1]
2. *Cyprinodon variegatus* (sheepshead minnow) (Table 17).
3. *Menidia beryllina* (inland silverside), *M. menidia* (Atlantic silverside), and *M. peninsulae* (tidewater silverside) (Table 18).

6.1.3 The test species (AFS, 1991) listed in Subsection 6.1.2 are the recommended acute toxicity test organisms. They are easily cultured in the laboratory, are sensitive to a variety of pollutants, and are generally available throughout the year from commercial sources. Summaries of test conditions for these species are provided in Tables 12-18. Guidelines for culturing and/or holding the organisms are provided in Appendix A.

6.1.4 Additional species may be suitable for toxicity tests in the NPDES Program. A list of alternative acute toxicity test species and minimal testing requirements (i.e., temperature, salinity, and life stage) for these species are provided in Appendix B. Table 19 provides a summary of test conditions for *Holmesimysis costata*, which should also be considered an alternative acute toxicity test species. The *Holmesimysis costata* Acute Test (Table 19) is specific to Pacific Coast waters and is not listed at 40 CFR Part 136 for nationwide use. It is important to note that these species may not be as easily cultured or tested as the species on the list in 6.1.2, and may not be available from commercial sources.

6.1.5 Some states have developed culturing and testing methods for indigenous species that may be as sensitive or more sensitive than the species recommended in 6.1.2. However, EPA allows the use of indigenous species only where state regulations require their use or prohibit importation of the species in 6.1.2. Where state regulations prohibit importation or use of the recommended test species, permission must be requested from the appropriate state agency prior to their use.

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[1] The genus name of this organism was formally changed to *Americamysis* (Price *et al.*, 1994); however, the method manual will continue to refer to *Mysidopsis bahia* to maintain consistency with previous versions of the method.

6.1.6 Where states have developed culturing and testing methods for indigenous species other than those recommended in this manual, data comparing the sensitivity of the substitute species and one or more of the recommended species must be obtained in side-by-side toxicity tests with reference toxicants and/or effluents, to ensure that the species selected are at least as sensitive as the recommended species. These data must be submitted to the permitting authority (State or Region) if required. EPA acknowledges that reference toxicants prepared from pure chemicals may not always be representative of effluents. However, because of the observed and/or potential variability in the quality and toxicity of effluents, it is not possible to specify a representative effluent.

6.1.7 Guidance for the selection of test organisms where the salinity of the effluent and/or receiving water requires special consideration is provided in the Technical Support Document for Water Quality-Based Toxics Control (USEPA, 1991c).

1. Where the salinity of the receiving water is <1‰, freshwater organisms are used regardless of the salinity of the effluent.
2. Where the salinity of the receiving water is ≥1‰, the choice of organisms depends on state water quality standards and/or permit requirements.

6.2 **SOURCES OF TEST ORGANISMS**

6.2.1 INHOUSE CULTURES

6.2.1.1 Inhouse cultures should be established wherever it is cost effective. If inhouse cultures cannot be maintained, test organisms should be purchased from experienced commercial suppliers (see Appendix for sources).

6.2.2 COMMERCIAL SUPPLIERS

6.2.2.1 All of the principal test organisms listed in Subsection 6.1.2 are available from commercial suppliers.

6.2.3 FERAL (NATURAL OCCURRING, WILD CAUGHT) ORGANISMS

6.2.3.1 The use of test organisms taken from the receiving water has strong appeal, and would seem to be the logical approach. However, it is impractical for the following reasons:

1. Sensitive organisms may not be present in the receiving water because of previous exposure to the effluent or other pollutants.
2. It is often difficult to collect organisms of the required age and quality from the receiving water;
3. Most states require collection permits, which may be difficult to obtain. Therefore, it is usually more cost effective to culture the organisms in the laboratory or obtain them from private, state, or Federal sources. Fish such as fathead minnows, sheepshead minnows, and silversides, and invertebrates such as daphnids and mysids, are easily reared in the laboratory or purchased.
4. The required QA/QC records, such as the single laboratory precision data, would not be available.
5. Since it is mandatory that the identity of test organisms is known to the species level, it would necessary to examine each organism caught in the wild to confirm its identity, which would usually be impractical or, at the least, very stressful to the organisms.
6. Test organisms obtained from the wild must be observed in the laboratory for a minimum of one week prior to use, to assure that they are free of signs of parasitic or bacterial infections and other adverse effects. Fish captured by electroshocking must not be used in toxicity testing.

6.2.3.2 Guidelines for collection of feral organisms are provided in USEPA, 1973; USEPA 1990a.

6.2.4 Regardless of their source, test organisms should be carefully observed to ensure that they are free of signs of stress and disease, and in good physical condition. Some species of test organisms, such as trout, can be obtained from stocks certified as "disease-free."

6.3 LIFE STAGE

6.3.1 Young organisms are often more sensitive to toxicants than are adults. For this reason, the use of early life stages, such as first instars of daphnids and juvenile mysids and fish, is required for all tests. In a given test, all organisms should be approximately the same age and should be taken from the same source. Since age may affect the results of the tests, it would enhance the value and comparability of the data if the same species in the same life stages were used throughout a monitoring program at a given facility.

6.4 LABORATORY CULTURING

6.4.1 Instructions for culturing and/or holding the recommended test organisms are included in Appendix A.

6.5 HOLDING AND HANDLING TEST ORGANISMS

6.5.1 Test organisms should not be subjected to changes of more than 3EC in water temperature or 3‰ in salinity in any 12 h period.

6.5.2 Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress. Organisms that are dropped or touch dry surfaces or are injured during handling must be discarded. Dipnets are best for handling larger organisms. These nets are commercially available or can be made from small-mesh nylon netting, silk bolting cloth, plankton netting, or similar material. Wide-bore, smooth glass tubes (4 to 8 mm inside diameter) with rubber bulbs or pipettors (such as a PROPIPETTE® or other pipettor) should be used for transferring smaller organisms such as daphnids, mysids, and larval fish.

6.5.3 Holding tanks for fish are supplied with a good quality water (see Section 5, Facilities and Equipment) with a flow-through rate of at least two tank-volumes per day. Otherwise, use a recirculation system where the water flows through an activated carbon or undergravel filter to remove dissolved metabolites. Culture water can also be piped through high intensity ultraviolet light sources for disinfection, and to photodegrade dissolved organics.

6.5.4 Crowding should be avoided. The DO must be maintained at a minimum of 4.0 mg/L for marine and warm water, freshwater species, and 6.0 mg/L for cold-water, freshwater species. The solubility of oxygen depends on temperature, salinity, and altitude. Aerate if necessary.

6.5.5 Fish should be fed as much as they will eat at least once a day with live or frozen brine shrimp or dry food (frozen food should be completely thawed before use). Brine shrimp can be supplemented with commercially prepared food such as Tetramin® or BioRil® flake food, or equivalent. Excess food and fecal material should be removed from the bottom of the tanks at least twice a week by siphoning.

6.5.6 Fish should be observed carefully each day for signs of disease, stress, physical damage, and mortality. Dead and abnormal specimens should be removed as soon as observed. It is not uncommon to have some fish (5-10%) mortality during the first 48 h in a holding tank because of individuals that refuse to feed on artificial food and die of starvation.

6.5.7 A daily record of feeding, behavioral observations, and mortality should be maintained.

6.6 TRANSPORTATION TO THE TEST SITE

6.6.1 Organisms are transported from the base or supply laboratory to a remote test site in culture water or standard dilution water in plastic bags or large-mouth screw-cap (500 mL) plastic bottles in styrofoam coolers. Adequate DO is maintained by replacing the air above the water in the bags with oxygen from a compressed gas cylinder, and sealing the bags. Another method commonly used to maintain sufficient DO during shipment is to aerate with an airstone which is supplied from a portable pump. The DO concentration must not fall below 4.0 mg/L for marine and warm-water, freshwater species, and 6.0 mg/L for cold-water, freshwater species.

6.6.2 Upon arrival at the test site, organisms are transferred to receiving water if receiving water is to be used as the test dilution water. All but a small volume of the holding water (approximately 5%) is removed by siphoning, and replaced slowly over a 10 to 15 min period with dilution water. If receiving water is used as dilution water, caution must be exercised in exposing the test organisms to it, because of the possibility that it might be toxic. For this reason, it is recommended that only approximately 10% of the test organisms be exposed initially to the dilution water. If this group does not show excessive mortality or obvious signs of stress in a few hours, the remainder of the test organisms are transferred to the dilution water.

6.6.3 A group of organisms must not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed, or if mortality appears to exceed 10% preceding the test. If the organisms fail to meet these criteria, the entire group must be discarded and a new group obtained. The mortality may be due to the presence of toxicity, if receiving water is used as dilution water, rather than a diseased condition of the test organisms. If the acclimation process is repeated with a new group of test organisms and excessive mortality occurs, it is recommended that an alternative source of dilution water be used.

6.6.4 In static tests, marine organisms can be used at all concentrations of effluent by adjusting the salinity of the effluent to a standard salinity (such as 25‰) or to the salinity approximating that of the receiving water, by adding sufficient dry ocean salts, such as Forty Fathoms®, or equivalent, GP2 or hypersaline brine.

6.6.5 Saline dilution water can be prepared with deionized water or a freshwater such as well water or a suitable surface water. If dry ocean salts are used, care must be taken to ensure that the added salts are completely dissolved and the solution is aerated 24 h before the test organisms are placed in the solutions. The test organisms should be acclimated in synthetic saline water prepared with the dry salts. Caution: addition of dry ocean salts to dilution water may result in an increase in pH. (The pH of estuarine and coastal saline waters is normally 7.5-8.3.)

6.6.6 All effluent concentrations and the control(s) used in a test should have the same salinity. However, if this is impractical because of the large volumes of water required, such as in flow-through tests, the highest effluent concentration (lowest salinity) that could be tested would depend upon the salinity of the receiving water and the tolerance of the test organisms. The required salinities for toxicity tests with estuarine and marine species are listed in Tables 16-19. However, the tolerances of other candidate test species would have to be determined by the investigator in advance of the test.

6.6.7 Because of the circumstances described above, when performing flow-through tests of effluents discharged to saline waters, it is advisable to acclimate groups of test organisms to each of three different salinities, such as 10, 20, and 30‰, prior to transporting them to the test site. It may also be advisable to maintain cultures of these test organisms at a series of salinity levels, including at least 10, 20, and 30‰, so that the change in salinity upon acclimation at the desired test dilutions does not exceed 6‰.

6.7 **TEST ORGANISM DISPOSAL**

6.7.1 When the toxicity test is concluded, all test organisms (including controls) should be humanely destroyed and disposed of in an appropriate manner.